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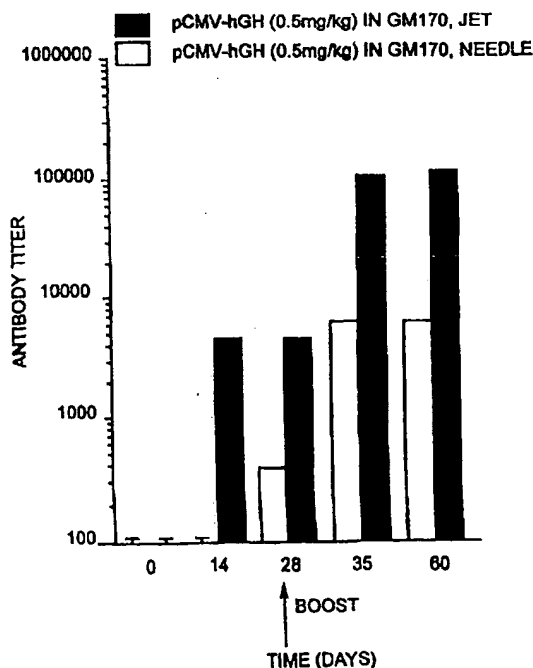
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(54) Title: NEEDLE-FREE INJECTION OF FORMULATED NUCLEIC ACID MOLECULES

(57) Abstract

A novel method is provided for delivering nucleic acid molecules through and/or to the skin of mammals by needle-free injection. The method involves the incorporation of formulated nucleic acid molecules with devices for injecting the molecules by air, fluid and/or mechanical pressure. Disclosed are compositions and methods for enhancing the administration to and uptake of nucleic acids in a mammal. The methods disclosed provide an increased immune response by allowing the uptake of formulated nucleic acid molecules by a wide variety of cell types simultaneously. Also disclosed are examples which demonstrate that the combination of formulated nucleic acid molecules and needle-free injection methods results in immune responses which are superior to those obtained by conventional means of delivery. Methods for delivery, as well as methods for formulating nucleic acid molecules with various compounds, such as cationic complexing agents, polymeric and non-polymeric formulations, protective, interactive, non-condensing systems are also disclosed.



DESCRIPTIONNEEDLE-FREE INJECTION OF FORMULATED NUCLEIC ACID MOLECULES5    Statement of Related Applications

          This application is related to U.S. Patent Application entitled "NUCLEIC ACID TRANSPORTERS FOR DELIVERY OF NUCLEIC ACIDS INTO A CELL" Serial Number 08/484,777, filed June 7, 1995, International Patent Application entitled "NUCLEIC ACID TRANSPORTERS FOR DELIVERY OF NUCLEIC ACIDS INTO A  
10   CELL" No. PCT/US96/05679 filed April 23, 1996 and U.S. Patent Application "TRANSPORTERS FOR SPECIFIC DELIVERY OF MACROMOLECULES TO CELLS" Serial Number 60/045,295, filed May 2, 1997 all of which are incorporated herein by reference in their entirety, including any drawings.

15   Introduction

          The present invention relates to products and methods useful for delivering formulated nucleic acid molecules by needle-free injection.

Background of the Invention

20       The following information is presented solely to assist the understanding of the reader, and none of the information is admitted to describe or constitute prior art to the claims of the present invention.

          In the past, non-viral administration of nucleic acids *in vivo* has been pursued by a variety of methods. These include lipofectin/liposome fusion: *Proc. Natl. Acad. Sci.*,  
25   Volume 84, pp. 7413-7417 (1993); polylysine condensation with and without adenovirus enhancement: *Human Gene Therapy*, Volume 3, pp. 147-154 (1992); and transferrin:transferrin receptor delivery of nucleic acid to cells: *Proc. Natl. Acad. Sci.*, Volume 87, pp. 3410-3414 (1990). The use of a specific composition consisting of polyacrylic acid has been disclosed in WO 94/24983. Naked DNA has been administered  
30   as disclosed in International Patent Publication No. WO 90/11092.

Since the introduction of the needle-syringe, injection technology has remained virtually unchanged. Once considered a simple and innocuous part of medical therapy, injections now take on ominous consequences. Needlestick injuries can be a life threatening event. Jet injection, or the injection of proteins by aerosol pressure, was introduced into clinical use in 1947 (*Military Medicine*, June, 516-524, 1963), and a base of scientific data has validated the safety and efficacy of this method of injection (*New Zealand Medical Journal*, Vol. 95. p.815, 1983). Current aerosol pressure injectors are calibrated to allow the user to select the accurate depth of penetration, and are available with syringes designed for intramuscular, subcutaneous or epidermal injections.

Use of aerosol pressure injections to immunize rabbits to HbsAg protein has been shown to be superior to classical syringe and needle methods, and resulted in a nearly 4 fold increase in antibody production at eight weeks when compared with a normal syringe and needle (Davis et al., *Vaccine* vol.12(16):1503-9 1994). In the Davis et al., study the DNA was suspended in endotoxin-free Dulbeccos's PBS and the area to be injected was not pretreated.

The transfection of tissue by aerosol injection has been reported as being superior to needle injection methods (Kerr et al., *Journal of Cellular Biochemistry Supplement* 0 (21A). 1995). It has also been shown that aerosol injection of rabbit papilloma virus genome into rabbit epithelium results in the induction of papillomas (Brandsma et al., *Proc. natl. Acad. Sci. USA* 88. 4816-4820. 1991). Aerosol injection has been used to introduce human cytomegalovirus immediate early gene 1 enhancer/ promoter sequences, bacterial chloramphenicol acetyltransferase, whey acidic protein promoter sequences, and the bacterial -galactosidase gene through the skin surface so as to transfect skin, muscle, fat and mammary tissue of living animals (Furth et al., *Analytical biochemistry*, 205, 365-368, 1992). It has been stated that suspending aerosol-injected DNA constructs in solutions which enhance cellular uptake of DNA may increase the number of cells transfected in a single injection (Furth et al., *supra*).

DNA bound by precipitation methods to inert particles have been delivered by needle-free injection. Coating inert particles, such as gold microprojectiles, and injecting them by needle-free devices offers direct injection into the cells due to the projectile carrier penetrating the cell. Tang et al., inoculated mice with DNA encoding human

growth hormone coated gold microprojectiles and stated that this method was superior to needle injection of plasmids encoding human growth hormone. Tang et al, reports that this technique simplifies the procedure and shortens the time required to produce antibodies to particular proteins by eliminating steps for protein purification and adjuvant administration. (Tang et al., Nature, Vol. 356:152-154 1992)

#### Summary Of the Invention

This invention features compositions and methods for enhancing the administration to and uptake of nucleic acids in a mammal. An efficient strategy for enhancing needle-free delivery of nucleic acids *in vivo* is to protect the nucleic acid from degradation, thereby maintaining the administered nucleic acid at the target site in order to further increase its cellular uptake. The data presented herein demonstrates that the combination of formulated nucleic acid molecules and needle free injection methods are synergistic, providing the desired antibody response to the resulting expressed protein that is unexpectedly high when compared with either needle-free injection of non-formulated nucleic acids or needle injection of formulated nucleic acids.

The invention provides a method to deliver formulated nucleic acid molecules through and/or to the skin of a mammal by using an apparatus configured and arranged to administer molecules by air, or mechanical pressure through and/or to the skin of a mammal. Thus, the present invention allows for superior delivery of nucleic acid molecules into cells *in vivo* by the combination of a needle free device and formulated nucleic acid molecules. Furthermore, the present invention also allows for treatment of diseases, vaccination, and treatment of muscle disorder and serum protein deficiencies.

In a first aspect, the present invention features a method for delivering a nucleic acid molecule formulated with a transfection facilitating agent through and/or to the skin of a mammal by the use of a needle-free injection device. Preferably, the needle-free device is configured and arranged to cause aerosol delivery of the formulated nucleic acid through and/or to the skin of the mammal.

By "delivery" or "delivering" is meant transportation of nucleic acid molecules to desired cells or any cells. The nucleic acid molecules will be delivered to multiple cell lines, including the desired target. Delivery results in the nucleic acid molecules coming

in contact with the cell surface, cell membrane, cell endosome, within the cell membrane, nucleus or within the nucleus, or any other desired area of the cell from which transfection can occur within a variety of cell lines which can include but are not limited to; epithelial cells, Langerhan cells, Langhans' cells, littoral cells, keratinocytes, dendritic cells,  
5 macrophage cells, kupffer cells, lymphocytes and lymph nodes. Preferably, the nucleic acid molecule can be delivered through and/or to the skin by aerosol pressure and is not significantly sheared.

The term "nucleic acid" as used herein refers to both RNA and DNA including: cDNA, genomic DNA, plasmid DNA or condensed nucleic acid, nucleic acid formulated  
10 with cationic lipids, nucleic acid formulated with peptides, antisense molecule, cationic substances, RNA or mRNA. In a preferred embodiment, the nucleic acid administered is plasmid DNA which comprises a "vector". The nucleic acid can be, but is not limited to, a plasmid DNA vector with a eukaryotic promoter which expresses Human Growth Hormone, such as in the example provided.

15 The term "formulated" as used herein means the process by which nucleic acid molecules are combined with a transfection facilitating agent in a manner which makes the nucleic acid molecules more stable, protected, and therefore, more easily transferable.

The term "transfection facilitating agent" as used herein refers to an agent that forms a complex with the nucleic acid. This molecular complex is associated with nucleic  
20 acid, molecule in either a covalent or a non-covalent manner. The transfection facilitating agent should be capable of transporting nucleic acid molecules in a stable state and of releasing the bound nucleic acid molecules into the cellular interior. The transfection facilitating agent should also be capable of being bound to nucleic acid molecules and lyophilized or freeze dried and either rehydrated prior to needle-free delivery or delivered  
25 as a fine powder via needle-free delivery.

In addition, the transfection facilitating agent may prevent lysosomal degradation of the nucleic acid molecules by endosomal lysis. Furthermore, the transfection facilitating agent allows for efficient transport of the nucleic acid molecule through the cytoplasm of the cell to the nuclear membrane and into the nucleus and provide protection.

30 In a preferred embodiment transfection facilitating agents are non-condensing polymers, oils and surfactants. These may be suitable for use as compounds which

prolong the localized bioavailability of a nucleic acid: polyvinylpyrrolidones; polyvinylalcohols; propylene glycols; polyethylene glycols; polyvinylacetates; poloxamers (Pluronics)(block copolymers of propylene oxide and ethylene oxide, relative amounts of the two subunits may vary in different poloxamers); poloxamines (Tetronics); ethylene vinyl acetates; celluloses, including salts of carboxymethylcelluloses, methylcelluloses, hydroxypropylcelluloses, hydroxypropylmethylcelluloses; salts of hyaluronates; salts of alginates; heteropolysaccharides (pectins); dextrans; chitosans; phosphatidylcholines (lecithins); miglyols; polylactic acid; polyhydroxybutyric acid. Some of these compounds may be used as protective, interactive, non-condensing compounds and others as sustained release compounds, while some may be used in either manner under the respectively appropriate conditions.

In another embodiment cationic condensing agents such as cationic lipids, peptides, or lipopeptides may associate with the nucleic acid molecule and may facilitate transfection.

In a further embodiment some of these compounds may be covalently attached to gold particles and thereby bind with nucleic acid molecules of the present invention. Gold particles coated with a polymer or polymers of the present invention can deliver nucleic acid molecules into cells by penetrating the cell when delivered by needle-free injection device.

The term "protects" or "protective" or "protected" as used herein refers to an effect of the interaction between such a compound and a nucleic acid such that the rate of degradation of the nucleic acid is decreased in a particular environment, thereby prolonging the localized bioavailability of the nucleic acid molecule. Such degradation may be due a variety of different of factors, which specifically include the enzymatic action of a nuclease. The protective action may be provided in different ways, for example, by exclusion of the nuclease molecules or by exclusion of water.

The compounds which protect the nucleic acid and/or prolong the localized bioavailability of a nucleic acid may achieve one or more of the following effects, due to their physical, chemical or rheological properties: (1) Protect nucleic acid, for example plasmid DNA, from nucleases due to steric, viscosity, or other effects such as shearing; (2) increase the area of contact between nucleic acid, such as plasmid DNA, through extracellular

matrices and over cellular membranes, into which the nucleic acid is to be taken up; (3) concentrate nucleic acid, such as plasmid DNA, at cell surfaces due to water exclusion; (4) indirectly facilitate uptake of nucleic acid, such as plasmid DNA, by disrupting cellular membranes due to osmotic, hydrophobic or lytic effects; and (5) indirectly facilitate uptake  
5 of nucleic acids by allowing diffusion of protected nucleic acid chains through tissue at the administration site.

By "prolonging the localized bioavailability of a nucleic acid" is meant that a nucleic acid when administered to an organism in a composition comprising such a compound will be available for uptake by cells for a longer period of time than if  
10 administered in a composition without such a compound, for example when administered in a saline solution. This increased availability of nucleic acid to cells could occur, for example, due to increased duration of contact between the composition containing the nucleic acid and a cell or due to protection of the nucleic acid from attack by nucleases. The compounds which prolong the localized bioavailability of a nucleic acid are suitable  
15 for internal administration.

By "suitable for internal administration" is meant that the compounds are suitable to be administered within the tissue of an organism, for example within a muscle or within a joint space, epidermally, intradermally or subcutaneously. Properties making a compound suitable for internal administration can include, for example, the absence of a  
20 high level of toxicity to the organism as a whole.

The term "needle-free injection device" as used herein relates to an apparatus that is capable of injecting an aerosol through and/or to the skin of a mammal into the tissue by air and/or mechanical pressure. It is understood that conventional devices of this type are calibrated to allow one of ordinary skill in the art to select and/or adjust the desired  
25 injection depth and therefore it is expected that future devices that perform this function will also be calibrated in the same manner. It is also understood that devices of this type may have a needle which is only used to collect a solution which is subsequently aerosolized, and delivered by needle-free means. The type of injection device is not considered a limiting aspect of the present invention. The primary importance of a needle-  
30 free device is, in fact, the capability of the device to deliver an aerosol of formulated nucleic acid molecules through and/or to the skin of a mammal. The needle-free injection

device can include, for example, a Gene Gun or a Needle-Less Injector as described in U.S. Patent 5,480,381 or a powder delivery device such as in PCT WO/O97/134652.

The term "apparatus" as used herein relates to the set of components that upon combination allow the delivery of an aerosol through and/or to the skin of a mammal. The components can be a nozzle or needle-free syringe with which one can collect and/or administer the formulated nucleic acid molecules, and a pump or spring for creating air pressure which forcibly evacuates the formulated nucleic acid molecules from the nozzle or needle-free syringe in a manner that creates an aerosol capable of penetrating the skin of a mammal. To create air pressure the apparatus can employ gas pressure, gas spring or spring force.

Preferably, the apparatus is capable of being calibrated to allow selection of depth of delivery. Hence, delivery can occur through the skin or to the skin.

The term "aerosol" as used herein is a suspension of formulated nucleic acid molecules in the form of a particulate mist. Aerosols have been defined as colloidal systems consisting of very finely subdivided liquid or solid particles dispersed in and surrounded by a gas. The aerosol of the present invention can depend upon the power of a liquified or compressed gas or mechanical spring to generate the fine mist of formulated nucleic acid molecules. Particles of an aerosol can range from less than 1 to 50 m. The particles are said to remain suspended in the air for relatively long periods of time. The size of the particles can be measured by conventional methods such as the Milken Oil Drop Experiment for measuring aerosol particle size, but the need to determine specific size is falling into disuse (Sciarra, et al., in Remington's Pharmaceutical Sciences, 18th ed. chapter 92, 1990). The aerosol can be a liquid, a powder, or a heterogeneous mist comprising both a liquid and solid phase.

The term "skin" refers to the outer covering of a mammal consisting of epidermal and dermal tissue and appendages such as sweat ducts and hair follicles. Skin can comprise the hair of a mammal in cases where the mammal has an epidermis which is covered by hair. In mammals which have enough hair to be considered fur or a pelt it is preferable to shave the hair, leaving primarily skin.

The term "mammal" refers to any warm blooded organism. Preferably the mammal is a human.



In preferred embodiments the method results in an immune response, preferably a humoral immune response targeted for the protein product encoded by the nucleic acid molecule, such as an antibody response that is preferably at least 3 times greater than an antibody response caused by needle injection of a protein product encoded by a nucleic acid molecule suspended in saline, and at least 10 times greater than an antibody response  
5 caused by needle injection of a nucleic acid molecule formulated with a transfection facilitating agent. In other situations the immune response preferably is a cytotoxic T-lymphocyte response.

The term "immune response" as used herein refers to the mammalian natural  
10 defense mechanism which can occur when foreign material is internalized. The immune response can be a global immune response involving the immune system components in their entirety. Preferably the immune response results from the protein product encoded by the formulated nucleic acid molecule. The immune response can be, but is not limited to; antibody production, T-cell proliferation /differentiation, activation of cytotoxic T-  
15 lymphocytes, and/or activation of natural killer cells. Preferably the immune response is a humoral immune response. However, as noted above, in other situations the immune response, preferably, is a cytotoxic T-lymphocyte response.

The term "humoral immune response" refers to the production of antibodies in response to internalized foreign material. Preferably the foreign material is the protein  
20 product encoded by a formulated nucleic acid molecule internalized by injection with a needle free device.

In an additional embodiment the needle-free device is selected from the group consisting of: Mediject, Bioject, Gene Gun, Mesoflash, Ped-O-ject and Powder-Ject. Generally, it is understood that such a device is accompanied by directions for usage.

25 In another aspect the invention features a kit. The kit includes a provider for providing a nucleic acid molecule formulated with a transfection facilitating agent and a needle-free means for delivering the nucleic acid molecule through and/or to the skin of a mammal.

The "provider" can be instructions furnished to allow one of ordinary skill in the  
30 art to make formulated nucleic acid molecules. The instructions will furnish steps to make the compounds used for formulating nucleic acid molecules. Additionally, the instructions

will include methods for testing the formulated nucleic acid molecules that entail establishing if the formulated nucleic acid molecules are damaged upon injection from the needle-free device. The provider can also be the formulated nucleic acid molecules themselves.

5           The term "transfection" as used herein refers to the process of introducing DNA (e.g., formulated DNA expression vector) into a cell, thereby, allowing cellular transformation. Following entry into the cell, the transfected DNA may: (1) recombine with that of the host; (2) replicate independently as a plasmid or temperate phage; or (3) be maintained as an episome without replication prior to elimination.

10           As used herein, "transformation" relates to transient or permanent changes in the characteristics (expressed phenotype) of a cell induced by the uptake of a vector by that cell. Genetic material is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products.

Transformation of the cell may be associated with production of a variety of gene  
15           products including protein and RNA. These products may function as intracellular or extracellular structural elements, ligands, hormones, neurotransmitters, growth regulating factors, enzymes, chemotaxins, serum proteins, receptors, carriers for small molecular weight compounds, drugs, immunomodulators, oncogenes, cytokines, tumor suppressors, toxins, tumor antigens, antigens, antisense inhibitors, triple strand forming inhibitors,  
20           ribozymes, or as a ligand recognizing specific structural determinants on cellular structures for the purpose of modifying their activity. This list is only an example and is not meant to be limiting.

In another aspect, the invention features a method for making a kit. Preferably the method involves the step of combining a provider for providing a nucleic acid formulated  
25           with a transfection facilitating agent and a needle-free means for delivering the nucleic acid to a mammal. The provider for providing a nucleic acid can be instructions for formulating nucleic acid molecules or simply the formulated nucleic acid molecules.

In yet another aspect, the invention also features a method for treating a mammal that is suffering from a disorder conventionally treated by administering human growth  
30           hormone. The method requires administering a nucleic acid molecule encoding human

growth hormone and formulated with a transfection facilitating agent through and/or to the skin of the mammal by use of a needle-free device.

In another aspect, the invention features a method for treating a mammal that is suffering from a cancer by administering a nucleic acid molecule encoding the appropriate cancer antigen. The method requires administering a nucleic acid molecule encoding a cancer antigen and formulated with a transfection facilitating agent through and/or to the skin of the mammal by use of a needle-free device.

Preferably, the cancer is melanoma and the appropriate cancer antigen is MAGE 1.

In yet another aspect, the invention also features a method for treating a mammal that is suffering from an infectious disease by administering a nucleic acid molecule encoding an antigen for the infectious disease. The method requires administering a nucleic acid molecule encoding an antigen for the infectious disease and formulated with a transfection facilitating agent through and/or to the skin of the mammal by use of a needle-free device.

In a preferred embodiment the infectious disease is chronic hepatitis and the antigen is HBV core antigen.

Administration as used herein refers to the route of introducing the formulated nucleic acid molecules of the invention into the body of cells or organisms. Administration includes the use of aerosol pressure as provided by a needle free device to targeted areas of the mammalian body such as the muscle and the lymph nodes.

Prior to administration, the nucleic acid molecules of the invention can be formulated with at least one other type of molecule. For example, the molecular complexes can be formulated with other molecules such as polyvinyl-pyrrolidone as described herein. Formulation techniques are provided herein by example.

In a preferred embodiment administration is directed through the epidermis, intradermis, and subcutaneous layer to the muscle tissue. The present invention administers formulated nucleic acid molecules in a manner which causes contact with various mammalian cell types which are not contacted by conventional needle injection techniques during one injection. Cell lines contacted by injection with the present invention are, but are not limited to, epithelial cells, langerhan cells, keratinocytes, dendritic cells, macrophage cells, kupfer cells, lymphocytes and lymph nodes.

Another aspect of the present invention features a method for modifying inert particles by first washing the inert particles in fuming nitric acid solutions or in solutions of nitric acid mixed with sulfuric acid, and then combining the inert particles with cysteine terminated cationic DNA binding peptides to form a monolayer peptide coating.

- 5 Preferably, the DNA binding peptide recognizes and binds a specific nucleic acid sequence of a designed plasmid. Hence, one molecule of DNA binding peptide binds with an inert particle and a plasmid, therefore reducing the amount of crosslinking between inert particles. The inert particles can be but are not limited to gold particles. Preferably, the inert particles are combined with nucleic acid molecules and delivered through the skin of  
10 a mammal to cells by a needle-free device.

- The term "inert particles" refers to biologically inactive particles which can be but are not limited to particles from the group consisting of, ferrite crystals, gold particles or beads, tungsten spheres, other metals or biologically inactive compounds which are of a density of roughly  $15 - 20\text{g/cm}^3$  and a size of roughly  $1-3 \text{ m}$ . Generally, the optimum  
15 particle is small enough to produce minimal cell damage. The size of particles is not a limiting aspect. It is known by those of ordinary skill in the art that the particle must be large enough to acquire sufficient momentum to penetrate the cell: momentum being a function of size density and velocity, and by convention particle size should be roughly 10 times smaller than the target cell.

- 20 A further aspect of the invention features a method for delivering a nucleic acid molecule formulated with a modified inert particle through and/or to the skin of a mammal by the use of a needle-free injection device. Preferably, the needle-free device is configured and arranged to cause aerosol delivery of the formulated nucleic acid through and/or to the skin of the mammal.

- 25 The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the invention and from the claims.

#### Brief Description of The Drawings

- 30 The drawings will herein briefly be described.

Figure 1 shows the humoral immune response to hGH in dogs after needle and needle-free injection of transfection facilitating agent formulated pCMV-hGH in dogs. The antibody titer for each data point is an average for two dogs.

Figure 2 shows a comparison between pCMV-hGH suspended in saline and transfection facilitating agent formulated pCMV-hGH for the elicitation of humoral immune response to hGH in dogs after needle-free injection. The antibody titer for each data point is an average for two dogs.

Figure 3 shows the amounts of expressed Luciferase in conditions where a plasmid encoding CMV-Luciferase is formulated with modified gold particles that are coated or uncoated with Cys-Tyr-Lys-ala- (Lys)<sub>8</sub>-Trp-Lys (CK8). The formulations are then loaded on a Kapton carrier membrane in ethanol, water or precipitated with Ca<sup>2+</sup> and then loaded in ethanol.

#### Detailed Description of the Preferred Embodiments

The delivery of formulated nucleic acid molecules by the use of a needle free device represents a novel approach to gene delivery. The present invention offers a nucleic acid delivery apparatus that provides an increased immune response when compared to previous methods. The invention provides the advantage of allowing the uptake of formulated nucleic acid molecules by a wide variety of cell types simultaneously. Injecting formulated nucleic acid molecules by needle free device results in the formulated nucleic acid molecules directly contacting many more cell types than in conventional needle injection. Thus, the present invention provides an enhanced delivery of nucleic acid molecules and also provides a more efficient gene delivery system which can be used to generate an immune response.

Needle-free delivery of formulated nucleic acid molecules through and/or to the skin of a mammal, depends on several factors which are discussed below, including transfection efficiency and the composition of the formulated nucleic acid molecule.

#### I. DNA Injection Variables

The intensity of an immune response and the level of gene delivery and expression achieved with the present invention can be optimized (>5-fold effect over controls) by

altering the following variables. The variables are: the formulation (composition, plasmid topology), the technique and protocol for injection (angle of injection, state of muscle), and, the pretreatment of the muscle with myotoxic agents. An immune response can be measured by, but is not limited to, the amount of antibodies produced for a protein encoded and expressed by the injected nucleic acid molecule.

Other injection variables that can be used to significantly effect the levels of antibodies and/or cytotoxic T-lymphocytes produced in response to the protein encoded by the formulated nucleic acid molecule provided by the needle-free injection method of the present invention are the state of the muscle being injected and injection technique.

Examples of the variables include muscle stimulation, muscle contraction, muscle massage, delivery angle, and apparatus manipulation. Massaging the muscle may force plasmid out of the muscle either directly or via lymphatic drainage. By altering the depth of penetration and/or the angle at which the needle-free device is placed in relation to muscle fibers the present invention improves the plasmid distribution throughout the injection area which subsequently increases the antibody response to the protein which is encoded and expressed by the plasmid.

Needle free injection systems provide an attractive method for administration of plasmid DNA for the purpose of intramuscular immunization. Not only do they provide the general benefit of avoiding needle-stick injury, but they may produce better distribution of injected substances in the muscle. Direct gene transfer was first demonstrated on mammary tissue using the Ped-O-jet system, but this apparatus was designed largely for intradermal or subcutaneous injections (Furth et al., *Anal. biochem.* 205:365-368., 1992).

We have found that needle-free injection of formulated nucleic acids can be superior to classical needle injection in a dog model. We injected a plasmid, suspended in saline, containing the gene for Human Growth Hormone by needle-free and needle injection to dog muscle and measured antibody production. We found that with needle free injection at 14 and 28 days and prior to boost resulted in a measurable antibody titer versus needle injection which resulted in an almost undetectable titer.

## II. Nucleic acid based vaccines

The present invention can be used to deliver nucleic acid vaccines in a more efficient manner than is conventionally done at the present time. Nucleic acid vaccines, or the use of plasmid encoding antigens or therapeutic molecules such as Human Growth

5 Hormone, has become an area of intensive research and development in the last half decade. Comprehensive reviews on nucleic acid based vaccines have been published [M.A. Liu, et al.(Eds.), 1995, *DNA Vaccines: A new era in vaccinology*, Vol. 772, Ann. NY. Acad. Sci., New York; Kumar, V., and Sercarz, E., 1996, *Nat. Med.* 2:857-859; Ulmer, J.B., et al., (Eds.) *Current Opinion in Immunology*; 8:531-536. Vol. 772, Ann. NY.

10 Acad. Sci., New York]. Protective immunity in an animal model using plasmid encoding a viral protein was first observed in 1993 by Ulmer et al. [Ulmer, J.B., et al., 1993, *Science* 259:1745-1749]. Since then, several studies have demonstrated protective immunity for several disease targets and human clinical trials have been started. Many disease targets have been investigated. Examples include antigens of *Borrelia burgdorferi*, the tick-borne

15 infectious agent for Lyme disease (Luke et al., *J. Infect. Dis.* 175:91-97, 1997), human immunodeficiency virus-1, (Letvin et al., *Proc. Nat. Acad. Sci. USA* 94:9378-9383, 1997), B cell lymphoma (Syrengeles et al., *Nature Medicine.* 2:1038-41, 1996), Herpes simplex virus (Bourne et al., *J. Infectious dis.* 173:800-807, 1996), hepatitis C virus (Tedeschi et al., *Hepatology* 25:459-462, 1997), rabies virus (Xiang et al., *virology*, 209:569-579,

20 1995), *Mycobacterium tuberculosis* (Lowrie in Genetic Vaccines and Immunotherapeutic Strategies CA Thibeault, ed. Intl Bus Comm, Inc., southborough, MA 01772 pp. 87-122, 1996), and *Plasmodium falciparum* (Hoffman et al., *Vaccine* 15:842-845, 1997).

An important goal of gene therapy is to effect the uptake of nucleic acid by cells, thereby causing an immune response to the protein encoded by the injected nucleic acid.

25 Uptake of nucleic acid by cells is dependent on a number of factors, one of which is the length of time during which a nucleic acid is in proximity to a cellular surface. The present invention provides formulations which increase the length of time during which a nucleic acid is in proximity to a cellular surface, and penetrate the cell thereby delivering nucleic acid molecules into the cell.

30 Nucleic acid based vaccines are an attractive alternative vaccination strategy to subunit vaccines, purified viral protein vaccines, or viral vector vaccines. Each of the

traditional approaches has limitations that are overcome if the antigen(s) is expressed directly in cells of the body. Furthermore, these traditional vaccines are only protective in a strain-specific fashion. Thus, it is very difficult, and even impossible using traditional vaccine approaches to obtain long lasting immunity to viruses that have several sera types.  
5 or viruses that are prone to mutation.

Nucleic acid based vaccines offer the potential to produce long lasting immunity against viral epitopes that are highly conserved, such as with the nucleoprotein of viruses. Injecting plasmids encoding specific proteins by the present invention results in increased immune responses, as measured by antibody production. Thus, the present invention  
10 includes new methods of providing nucleic acid vaccines by delivering a formulated nucleic acid molecule with a needle-free device as described herein.

The efficacy of nucleic acid vaccines is enhanced by one of at least three methods: (1) the use of delivery systems to increase the stability and distribution of plasmid within the muscle, (2) by the expression (or delivery) of molecules to stimulate antigen  
15 presentation/transfer, or (3) by the use of adjuvants that may modulate the immune response.

### III. Polymeric and non-polymeric formulations for plasmid delivery to muscle

The present invention provides polymeric and non-polymeric formulations which  
20 address problems associated with injection of nucleic acids suspended in saline. Plasmids suspended in saline have poor bioavailability in muscle due to rapid degradation of plasmid by extracellular nucleases. One possible approach to overcome the poor bioavailability is to protect plasmid from rapid nuclease degradation by condensing the plasmid with commonly used cationic complexing agents. However, due to the  
25 physiology of the muscle, the use of rigid condensed particles containing plasmid for efficient transfection of a larger number of muscle cells has not been successful to date. Cationic lipid and polylysine plasmid complexes do not cross the external lamina to gain access to the caveolae and T tubules [Wolff, J.A., et al., 1992, *J. Cell. Sci.* 103:1249-1259].

30 Thus, the strategy identified for increasing the bioavailability of plasmid in muscle was to: protect plasmid from rapid extracellular nuclease degradation, disperse and retain



intact plasmid in the muscle, and facilitate the uptake of plasmid by muscle cells. Two specific methods of accomplishing this, which preferably are used in conjunction with needle-free delivery, are (a) the use of protective, interactive, non-condensing systems (b) the use of modified gold particles.

5           Delivery and expression of nucleic acids is limited due to degradation of the nucleic acids by components of organisms, such as nucleases. Thus, protection of the nucleic acids when delivered *in vivo* can greatly enhance the resulting expression, thereby enhancing a desired pharmacological or therapeutic effect. It was found that certain types of compounds which interact with a nucleic acid (e.g., DNA) in solution but do not  
10       condense the nucleic acid provide *in vivo* protection to the nucleic acid, and correspondingly enhance the expression of an encoded gene product. A detailed description of the formulations that can be used in the present invention can be found in PCT Application No. PCT/US96/05679 which is hereby incorporated as a reference in its entirety including any drawings. As noted above, preferably such formulations are  
15       delivered by using a needle free device as described herein.

          Inert particles coated with DNA have been used to deliver genes to cells. An advantage of coating inert particles, for instance gold beads, with nucleic acids is that the particle carrier actually penetrates the cell. Hence, the nucleic acid is delivered to the interior of the cell and should become incorporated and expressed in a more efficient  
20       manner. However, the conventional procedure for preparation of DNA-coated gold particles results in heterogeneous distribution of DNA from particle to particle, which produces variable expression from cell to cell (Butow et al., Meth Enzymol. 264:265-278, 1996).

          It is undesirable to create nucleic acid vaccines which produce variable results from sample to sample. Thus, the present invention provides gold particles uniformly coated  
25       with DNA by covalently attaching cysteine terminated DNA binding peptides to the gold surface. Furthermore, the invention enhances the intracellular transport of DNA released from the gold particles by non-covalent association of high affinity DNA binding peptides, which contain nuclear localization sequences, with the plasmid. The formulation provides  
30       the most reproducible amount of plasmid on gold particles, independent of the diameter of

the particle. Preferably such modified gold particle formulations are delivered by using a needle-free device as described herein.

#### VI. Diseases and Conditions for Intramuscular Plasmid Delivery

5           The present invention described herein can be utilized for the delivery and expression of many different coding sequences. In particular, the demonstrated effectiveness for the PINC systems (PCT Application No. PCT/US96/05679) for delivery to muscle indicate that such formulations are effective for delivery of a large variety of coding sequences to muscle by needle free injection. Specific suggestions for delivery of  
10 coding sequences to muscle with the needle free device of the present invention include those summarized in Table 1 below.

**Table 1: Applications for Plasmid-Based Gene Therapy  
15 by Intramuscular Injection**

Muscle and nerve disorders	References are numbered as they are cited in U.S. Application No. PCT/US96/05679, which has been incorporated by reference in its entirety.
20           Duchenne's muscular dystrophy Myotrophic disorders (IGF-I) Neurotrophic disorders (IGF-I)	Acsadi 1991 [5], Karpati 1993 [6], Miller 1995 [7] Coleman 1997 [8], Alila 1997 [9] Alila 1997 [9], Rabinovsky 1997 [10]
25           Secretion of expressed protein into the systemic circulation	
Hemophilias A and B [13]	Anwer 1996 [11], Kuwahara-Rundell 1994 [12], Miller 1994
30           Erythropoietin-responsive	Tripathy 1996 [14]
Pituitary dwarfism	Anwer 1996 [11], Dahler 1994 [15]
1-Antitrypsin deficiency	Levy 1996 [16]
Autoimmune and Inflammatory diseases	Raz 1993 [17]
Hypercholesterolemia	Fazio 1994 [18]
35           Hypotension	Ma 1995 [19]
Hypertension	Xiong 1995 [20]

	<b>Nucleic acid vaccines</b>	
	Herpes Simplex Virus	Manickan 1995 [21], Ghiasi 1995 [22], McClements 1996
	[23], Kriesel 1996 [24]	
5	Hepatitis B Virus	Davis 1993 [25], Davis 1994 [26], Davis 1996 [27]
	Influenza Virus	Donnelly 1995 [28], Ulmer 1993 [29], Ulmer 1994 [30]
	Tuberculosis	Lófwrie 1994 [31], Tascon, 1996 [32]
	Human Immunodeficiency Virus	Shiver 1995 [33], Coney 1994 [34], Wang 1993 [35]
	Cancer	Raz 1993 [17], Russell 1994 [36]
10	Malaria	Hoffman 1995 [37], Sedegah 1994 [38]
	Hepatitis C virus	Major 1995 [39], Lagging 1995 [40]
	Flavivirus	Phillipotts 1996 [41]
	Cytomegalovirus	Pande 1995 [42]
	Salmonella typhi	Lopez-Macias 1995 [43]
15	Mycoplasma pulmonis	Lai 1995 [44]
	Rabies virus	Xiang 1995 [45]

### Examples

20       The following examples are offered by way of illustration and are not intended to limit the scope of the invention in any manner. One of ordinary skill in the art would recognize that the various molecules and/ or amounts disclosed in the examples could be adjusted or substituted by larger amounts (for larger scaled experiments) or by inclusion of a different Transfection Facilitating Agent.

25

### **Example 1**

#### Demonstration of Transfection Facilitating Agent Plasmid DNA Complex Formation

#### Preparation of PVP Formulated Nucleic Acid Molecules

30       Concentrated pDNA stock solutions were made by lyophilizing and rehydrating pDNA with water to a final pDNA concentration of 3-5mg/ml. Formulations were made by aliquoting appropriate volumes of sterile stock solutions of pDNA, 5M NaCl, and polymer to obtain a final pDNA concentration in an isotonic polymer solution. Stock solutions were added in the following order: water, plasmid, polymer, and 5M NaCl. The

35       plasmid and polymers were allowed to incubate at room temperature for 15 minutes prior

to adding salt or lactose for ionicity adjustments. Likewise, Na-citrate buffers in 0.9% NaCl were added after incubating the plasmid and polymers for 15 minutes at room temperature. The osmotic pressure of selected formulations was measured (n=3) using a Fiske One-Ten Micro-Sample Osmometer. The pH of all formulations was measured using an Accumet Model 15 pH Meter and the viscosity of all formulations was measured using a Programmable Rheometer Model DV-III.

Dynamic dialysis was used with various interactive polymer formulations to measure binding between PVP and plasmid DNA. One ml of formulations and corresponding controls were placed in prewashed dialysis sacs. The dialysis sacs were closed and suspended in stirred saline solutions (100 ml) at 25°C. One ml aliquots were taken from the acceptor compartment over time and replaced with fresh media. The concentration of PVP in the diffused samples collected over time was measured spectroscopically at 220 nm.

In all cases, the rate of PVP diffusion through the dialysis membrane was decreased in the presence of plasmid DNA, indicating complex formation between PVP and plasmid DNA. The reduction in the diffusion rate for PVP in the presence of plasmid DNA was directly proportional to the initial amount of PVP in the dialysis sac. It was also determined that the sac volume remained constant during the duration of the experiment and that adherence of PVP to the membrane was negligible.

#### Preparation of Nucleic Acid Molecules Formulated With Modified Gold Particles

100 mg of 2.3 nm gold particles were placed in fuming nitric acid overnight at 23°C. The particles were then washed exhaustively with 18 Ω deionized water, which had been degassed under vacuum. A solution of Cys-Tyr-Lys-Ala-(Lys)<sub>8</sub>-Trp-Lys (CK8) was treated for 1 hr with 10mM dithiothreitol and then passed over a desalting column prepared in degassed water. Sufficient peptide to make the final solution 1mM CK8 was added to the cleaned gold particles and allowed to sit under N<sub>2</sub> overnight at 23°C. The particles were then washed with degassed water until there was no absorbance at 280 nm and then lyophilized. One µg of CMV-luciferase was added in water to 0.45 mg of modified gold particles, and then dried in a desiccator. Binding of the DNA to the CK8 modified particles was shown by eluting with 1.5 mM phosphate, and visualizing the

eluted relaxed and supercoiled plasmid on an agarose gel with ethidium bromide. As a control, DNA was precipitated with  $\text{Ca}^{++}$ , dried and added to the center of the Kapton carrier membrane in 100% ethanol. DNA formulated with modified gold particle was transferred to the Kapton membrane in water and in 100% ethanol. (Fig. 3)

5 Significant luciferase expression *in vivo* was observed with DNA formulated with modified gold particles when the formulation was transferred to the Kapton membrane in both 100% ethanol and, more importantly, in water (fig 3). Although the average expression was lower using water as the transfer solvent, this result suggests that it will be possible to include proteins such as transcription factors with the DNA.

10

### Example 2

#### Demonstration of Formulated Nucleic Acid Stability Upon Injection Through Needle Free Device.

Formulated plasmids were prepared as follows in a sterile manner in 2ml single dose  
15 vials.

Plasmid/Liposomes (DOTMA:DOPE 1:1 m/m)	1:3	-/+	20 g DNA/ml
Plasmid/K8/JTS-1	1:3:1	-/+/-	20 g DNA/ml
PVP 5%	w/v		20 g DNA/ml
20 PEG 10%	w/v		20 g DNA/ml
Control:			
DNA suspended in Saline			20 g DNA/ml

DNA formulations were injected by needle-free device into 50ml polypropylene  
25 tubes indicative of a "high impact" worst case scenario. Additionally the needle free device was set at maximum penetration as described in the product instruction manual.

DNA stability was determined by agarose gel electrophoresis of the samples before and after injection. Measurement of stability was determined by the amount of visible degradation, i.e. smearing, and by quantitating supercoiled and open circular DNA. Our  
30 data indicates that even in this extreme example the formulated DNA samples only exhibit slight degradation.

**Example 3****Demonstration of Formulated Nucleic Acid Stability Upon Injection Through Various Needle Free Device Delivery Nozzles**

Formulated nucleic acid molecules were prepared as follows in a sterile manner.

- 5 0.1 mg pCT0129/mL in DOTMA:Chol (1:1 m/m) 1:3 (-/+); 10%lactose  
3.0 mg pCT0129/mL in 5% PVP (50 kDa); 150 mM NaCl

Non-formulated samples:

- 0.1 mg pCT0129/mL in 150 mM NaCl  
3.0 mg pCT0129/mL in 150 mM NaCl

- 10 Each of these formulations were injected through different size needle-free injection nozzles. A non-injected formulation was used as a control for each experiment. The formulation was injected into a 50mL conical tube. Each sample was agarose gel electrophoresed after injection.

- Measurement of stability was determined by the amount of visible degradation, i.e.,  
15 smearing, and by quantitating supercoiled and open circular DNA. Our data indicates that even in this example which uses the most extreme injection parameters the nozzle size did not effect the state of the formulated DNA.

**Example 4**

- 20 **Elicitation of Immune Response Following Intramuscular Injection of plasmid.**

In this study, a Human Growth Hormone (hGH) expression plasmid is injected into the muscle of dogs and pigs to examine the production of anti-hGH antibodies.(figs 1,2).

- The expression plasmid was injected via a needle free device or by a needle to  
examine if the magnitude of the immune response is affected by the injection method or  
25 formulation.

- For elicitation of immune response, pCMV-hGH plasmid was suspended in saline or formulated with PVP and injected into biceps femoris and semitendinosus muscles of dogs using a needle free device or 22 gauge needle. Blood was collected before plasmid injection and once a week after the injection. Blood samples were kept overnight at 4° C,  
30 centrifuged at 2000 g for 15 min and serum was collected for detection of anti-hGH antibodies by ELISA.

Significant levels of hGH antibodies were detectable in jet (aerosol) injected animals 14 days after a single i.m. dose of hGH plasmid. The levels of hGH antibodies increased over time reaching a plateau by 21 days and remained elevated throughout the 112 day pre-boost periods. Repeating the plasmid dose on day 112 augmented the antibody response by 100 fold.

In comparison, the needle injection of hGH plasmid formulated with PVP did not produce significantly detectable levels of hGH antibodies. A booster dose was required to achieve significant antibody response from needle injection. Peak antibody levels from aerosol injections were about 20 times higher than from needle injections. Control animals received CMV-CAT plasmid formulated with PVP without the hGH gene, and the animals did not produce anti-hGH antibodies.

Needle-free injection of hGH plasmid suspended in saline also leads to the production of anti-hGH antibodies. A single dose of naked DNA was sufficient to achieve significant levels of hGH antibodies when administered via the needle-free device.

In comparison, multiple doses were required to elicit antibody response with needle injection. The antibody response from needle-free injection was 5 times higher compared to needle injection.

In the same experiment, needle-free injection gave 15-20 times better immune response compared to needle injection when the plasmid was formulated with PVP.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

5       The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of” and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as  
10 terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features,  
15 modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby  
20 described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Those references not previously incorporated herein by reference, including both  
25 patent and non-patent references, are expressly incorporated herein by reference for all purposes. Other embodiments are within the following claims.



CLAIMS:

1. A method for delivering a nucleic acid molecule to a mammal comprising the step of providing said nucleic acid molecule formulated with a transfection facilitating agent through and/or to the skin of said mammal by use of a needle-free device configured and arranged to cause aerosol delivery of said nucleic acid molecule through and/or to the skin of said mammal.  
5
2. The method of claim 1, wherein said nucleic acid molecule is DNA.  
10
3. The method of claim 1, wherein said nucleic acid molecule is a plasmid with a eukaryotic promoter which expresses a therapeutic molecule.
4. The method of claim 3, wherein said therapeutic molecule is for human growth hormone.  
15
5. The method of claim 1, wherein said nucleic acid molecule is RNA.
6. The method of claim 1, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.  
20
7. The method of claim 1, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more liposomes, one or more peptides, one or more modified gold particles, and one or more lipopeptides.  
25
8. The method of claim 1, wherein said method results in an antibody response.
9. The method of claim 8, wherein said antibody response is at least 3 times greater than the antibody response caused by needle injection of the nucleic acid molecule suspended in saline.  
30

10. The method of claim 9, wherein said antibody response is at least 10 times greater than the antibody response caused by needle injection of the nucleic acid molecule formulated with polyvinyl-pyrrolidone.

5

11. The method of claim 1, wherein said method induces an immune response.

12. The method of claim 11, wherein said immune response is a humoral immune response.

10

13. The method of claim 11, wherein said immune response is a T-cell mediated immune response.

14. The method of claim 11, wherein said immune response is a prophylactic immune response.

15

15. The method of claim 11, wherein said immune response is a therapeutic immune response.

20

16. The method of claim 1, wherein said mammal is a human.

17. The method of claim 1, wherein said needle-free means for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.

25

18. The method of claim 1, wherein said needle-free means for delivering is a gas pressure device.

19. The method of claim 1, wherein said needle-free means for delivering is a mechanical spring device.

30

20. The method of claim 1, wherein said needle-free means for delivering is a multi-port device.

21. A kit comprising a provider for providing a nucleic acid molecule formulated  
5 with a transfection facilitating agent and a needle-free device for delivering said nucleic acid molecule through and/or to the skin of a mammal.

22. The kit of claim 21, wherein said nucleic acid molecule is DNA.

10 23. The kit of claim 21, wherein said nucleic acid molecule is a plasmid with a eukaryotic promoter which expresses a gene.

24. The kit of claim 23, wherein said gene is human growth hormone.

15 25. The kit of claim 21, wherein said nucleic acid molecule is RNA.

26. The kit of claim 21, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.

20 27. The kit of claim 21, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more liposomes, one or more modified gold particles, one or more peptides, and one or more lipopeptides.

25 28. The kit of claim 21, wherein said needle-free device for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.

29. The kit of claim 21, wherein said needle-free device for delivering is a gas  
30 pressure device.

30. The kit of claim 21, wherein said needle-free device for delivering is a mechanical spring device.

31. The kit of claim 21, wherein said needle-free device for delivering is a multi-  
5 port device.

32. A method for making a kit comprising the steps of combining (i) a provider for providing a formulated nucleic acid molecule formulated with a transfection facilitating agent with (ii) a needle-free device for delivering said nucleic acid molecule through  
10 and/or to the skin of a mammal.

33. The method of claim 32, wherein said nucleic acid molecule is DNA.

34. The method of claim 32, wherein said nucleic acid molecule is a plasmid  
15 with a eukaryotic promoter which expresses a gene.

35. The method of claim 34, wherein said gene is for human growth hormone.

36. The method of claim 32, wherein said nucleic acid molecule is RNA.  
20

37. The method of claim 32, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.

38. The method of claim 32, wherein said transfection facilitating agent is  
25 selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more liposomes, one or more modified gold particles, one or more peptides, and one or more lipopeptides.

39. The method of claim 32, wherein said needle-free device for delivering is a  
30 needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.

40. The method of claim 32, wherein said needle-free device for delivering is a gas pressure device.

5 41. The method of claim 32, wherein said needle-free device for delivering is a mechanical spring device.

42. The method of claim 32, wherein said needle-free device for delivering is a multi-port device.

10

43. A method of treating a mammal suffering from a disorder conventionally treated by administering human growth hormone, comprising the step of providing a nucleic acid molecule formulated with a transfection facilitating agent encoding human growth hormone through and/or to the skin of said mammal by use of a needle-free device  
15 configured and arranged to cause aerosol delivery of said nucleic acid molecule through and/or to the skin of said mammal.

44. The method of claim 43, wherein said mammal is a human.

20 45. The method of claim 43, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.

46. The method of claim 43, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more  
25 cationic lipids, one or more liposomes, one or more modified gold particles, one or more peptides, and one or more lipopeptides.

47. The method of claim 43, wherein said needle-free device for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through  
30 and/or to the skin of a mammal.

48. The method of claim 43, wherein said needle-free device for delivering is a gas pressure device.

49. The method of claim 43, wherein said needle-free device for delivering is a mechanical spring device.

50. The method of claim 43, wherein said needle-free device for delivering is a multi-port device.

51. A method of treating a mammal suffering from cancer, comprising the step of providing a nucleic acid molecule formulated with a transfection facilitating agent through and/or to the skin of said mammal by use of a needle-free device configured and arranged to cause aerosol delivery of said nucleic acid molecule through and/or to the skin of said mammal, wherein said molecule encodes a cancer antigen.

52. The method of claim 51, wherein said mammal is a human.

53. The method of claim 51, wherein said cancer antigen is MAGE 1, and said cancer is melanoma.

54. The method of claim 51, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.

55. The method of claim 51, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more liposomes, one or more modified gold particles, one or more peptides, and one or more lipopeptides.

56. The method of claim 51, wherein said needle-free device for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.

57. The method of claim 51, wherein said needle-free device for delivering is a gas pressure device.

5 58. The method of claim 51, wherein said needle-free device for delivering is a mechanical spring device.

59. The method of claim 51, wherein said needle-free device for delivering is a multi-port device.

10

60. A method of treating a mammal suffering from an infectious disease, comprising the step of providing a nucleic acid molecule formulated with a transfection facilitating agent through and/or to the skin of said mammal by use of a needle-free device configured and arranged to cause aerosol delivery of said nucleic acid molecule through  
15 and/or to the skin of said mammal, wherein said molecule encodes an antigen for said infectious disease.

61. The method of claim 60, wherein said mammal is a human.

20 62. The method of claim 60, wherein said infectious disease antigen is HBV core antigen, and said infectious disease is chronic hepatitis.

63. The method of claim 60, wherein said transfection facilitating agent is a protective, interactive and non-condensing compound.

25

64. The method of claim 60, wherein said transfection facilitating agent is selected from the group consisting of: one or more polyvinyl-pyrrolidones, one or more cationic lipids, one or more liposomes, one or more modified gold particles, one or more peptides, and one or more lipopeptides.

30

65. The method of claim 60, wherein said needle-free device for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.

5 66. The method of claim 60, wherein said needle-free device for delivering is a gas pressure device.

67. The method of claim 60, wherein said needle-free device for delivering is a mechanical spring device.

10

68. The method of claim 60, wherein said needle-free device for delivering is a multi-port device.

69. A method of formulating nucleic acid molecules with modified inert particles  
15 comprising the steps of combining said nucleic acid molecules with said modified inert particles in an aqueous environment.

70. The method of claim 69, wherein said modified inert particles are modified gold particles.

20

71. A method of modifying gold particles comprising the steps of washing said gold particles in fuming or mixed acid solutions, and combining said gold particles with cationic DNA binding peptides to form a monolayer coating.

25 72. The method of claim 71, wherein said cationic DNA binding peptides have cysteine as the terminal residue.

73. The method of claim 71, wherein said fuming acid is nitric acid.

30 74. The method claim 71, wherein said mixed acid comprises nitric acid and sulfuric acid.



75. A method for delivering a nucleic acid molecule to a mammal comprising the step of providing said nucleic acid molecule formulated with modified gold particles through and/or to the skin of said mammal by use of a needle-free device configured and  
5 arranged to cause aerosol delivery through and/or to the skin of said mammal.

76. The method of claim 74, wherein said needle-free device for delivering is a needle-free device that injects said nucleic acid molecule by aerosol delivery through and/or to the skin of a mammal.  
10

77. The method of claim 74, wherein said needle-free device for delivering is a gas pressure device.

78. The method of claim 74, wherein said needle-free device for delivering is a  
15 mechanical spring device.

79. The method of claim 74, wherein said needle-free device for delivering is a multi-port device.

1/3

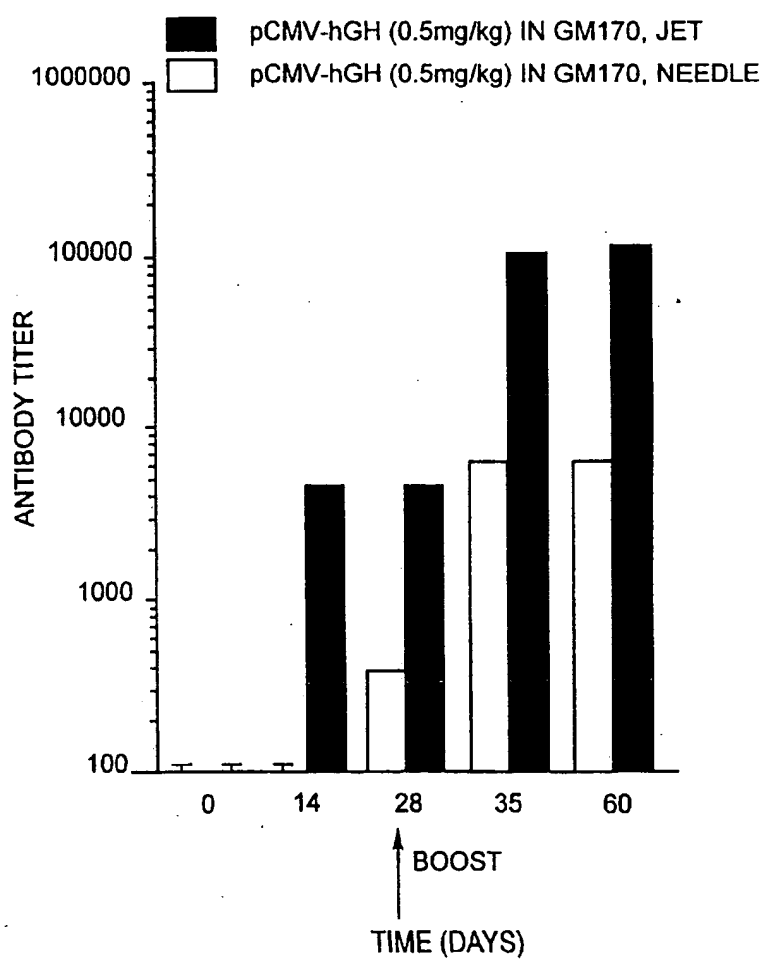


Fig. 1

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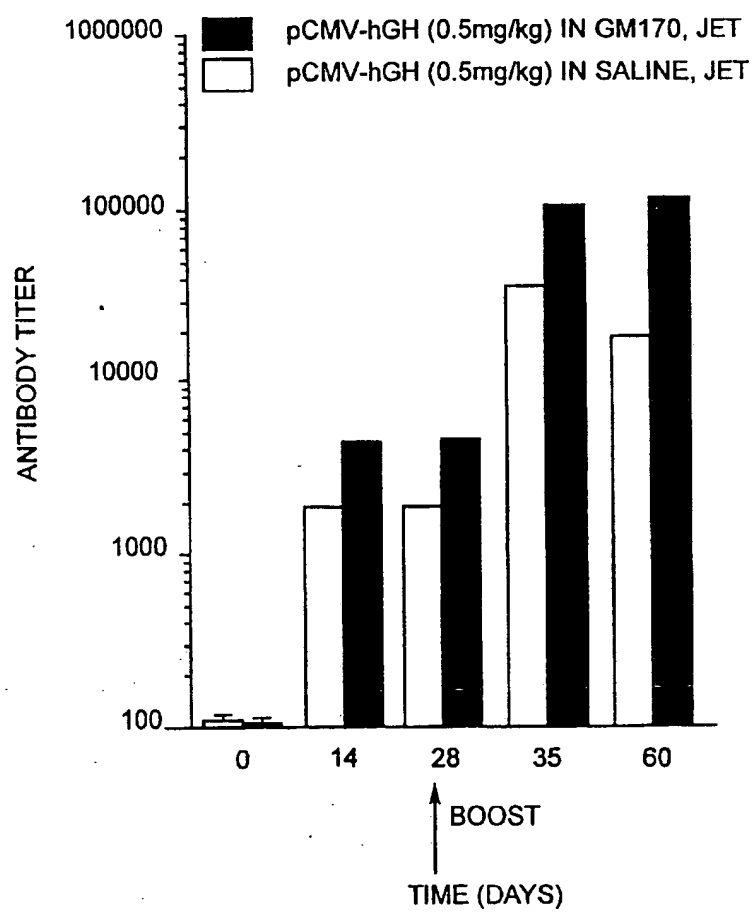


Fig. 2

3/3

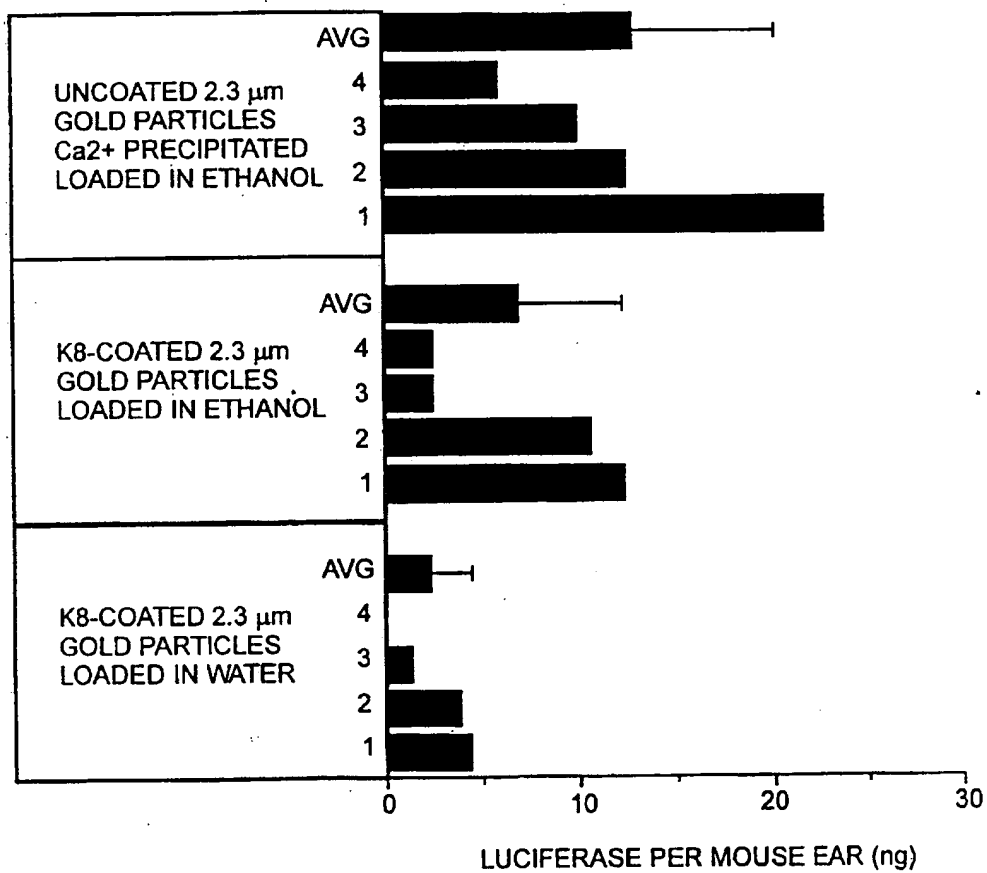


Fig. 3

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



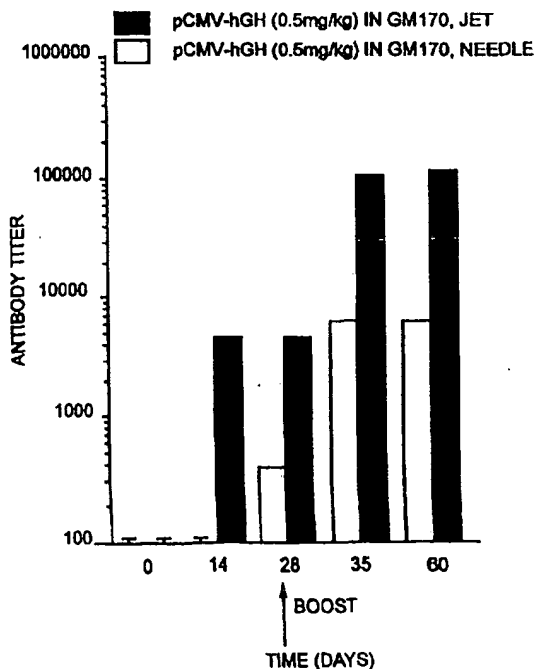
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(54) Title: NEEDLE-FREE INJECTION OF FORMULATED NUCLEIC ACID MOLECULES

(57) Abstract

A novel method is provided for delivering nucleic acid molecules through and/or to the skin of mammals by needle-free injection. The method involves the incorporation of formulated nucleic acid molecules with devices for injecting the molecules by air, fluid and/or mechanical pressure. Disclosed are compositions and methods for enhancing the administration to and uptake of nucleic acids in a mammal. The methods disclosed provide an increased immune response by allowing the uptake of formulated nucleic acid molecules by a wide variety of cell types simultaneously. Also disclosed are examples which demonstrate that the combination of formulated nucleic acid molecules and needle-free injection methods results in immune responses which are superior to those obtained by conventional means of delivery. Methods for delivery, as well as methods for formulating nucleic acid molecules with various compounds, such as cationic complexing agents, polymeric and non-polymeric formulations, protective, interactive, non-condensing systems are also disclosed.



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/26823

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/87 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FURTH, PRISCILLA A. (1) ET AL: "Gene Transfer into Mammalian Cells by Jet Injection." HYBRIDOMA, (1995) VOL. 14, NO. 2, PP. 149-152. ISSN: 0272-457X., XP002064168	1-3,8, 11-18, 21-23, 28,29, 32-34, 39,40
Y	see the whole document	4-7,9, 10,19, 20, 24-27, 30,31, 35-38, 41-68
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-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

8 April 1999

Date of mailing of the international search report

26. 07. 1999

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Hix, R

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/26823

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAYNES, JOEL R. (1) ET AL: "Induction and characterization of humoral and cellular immune responses elicited via gene gun-mediated nucleic acid immunization." ADVANCED DRUG DELIVERY REVIEWS, (1996) VOL. 21, NO. 1, PP. 3-18. ISSN: 0169-409X., XP002099106 see the whole document ---	1-68
X	BARRY, MICHAEL A. ET AL: "Biological features of genetic immunization." VACCINE, (1997) VOL. 15, NO. 8, PP. 788-791. ISSN: 0264-410X., XP002099107	1-3,8, 11-18, 21-23, 28,29, 32-34, 39,40
Y	see the whole document	4-7,9, 10,19, 20, 24-27, 30,31, 35-38, 41-68
X	BRANDSMA J L ET AL: "USE OF A RAPID, EFFICIENT INOCULATION METHOD TO INDUCE PAPILLOMAS BY COTTONTAIL RABBIT PAPILLOMAVIRUS DNA SHOWS THAT THE E7 GENE IS REQUIRED" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, June 1991, pages 4816-4820, XP002064169 cited in the application see the whole document ---	1-68
X	FURTH P A ET AL: "GENE TRANSFER INTO SOMATIC TISSUES BY JET INJECTION" ANALYTICAL BIOCHEMISTRY, vol. 205, September 1992, pages 365-368, XP000647725 cited in the application see the whole document ---	1-68
X	JOHNSTON S A ET AL: "GENE GUN TRANSFECTION OF ANIMAL CELLS AND GENETIC IMMUNIZATION" METHODS IN CELL BIOLOGY, vol. 43, 1 January 1994, pages 353-365, XP000575592 see page 361, paragraph 3 ---	1-68
Y	WO 94 24263 A (SARPHIE DAVID FRANCIS ;BELLHOUSE BRIAN JOHN (GB); GREENFORD JOHN C) 27 October 1994 see page 11, line 12 - line 24 ---	1-68
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# INTERNATIONAL SEARCH REPORT

Inter. nal Application No  
PCT/US 98/26823

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 20732 A (VIAGENE INC) 11 July 1996 see page 12, line 16 - page 14, line 10; example 5 ---	1-68
Y	WO 96 34967 A (UNIV LELAND STANFORD JUNIOR) 7 November 1996 see the whole document ---	1-68
X	D.E.KERR ET AL.: "Ovine mammary gland expression of jet-injected plasmid DNA: Northern blot and antibody analysis." JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, vol. 0, no. 21a, 1995, page 395 XP002099108 cited in the application see abstract ---	1-68
X	H.L. DAVIES ET AL.: "Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen." VACCINE, vol. 12, no. 16, 1994, pages 1503-1509, XP002099109 cited in the application see the whole document ---	1-68
P,X	LONGRIDGE, D. J. ET AL: "Effects of payload per unit area on dermal Powderject delivery of testosterone to conscious rabbits" PROC. INT. SYMP. CONTROLLED RELEASE BIOACT. MATER. (1998), 25TH, 595-596 CODEN: PCRMEY; ISSN: 1022-0178, 1998, XP002099110 see the whole document ---	1-68
P,X	ROGGE M.C. ET AL: "Impaired bioavailability of interferon beta-1a when administered intramuscularly by needle - free injection." DRUG DELIVERY: JOURNAL OF DELIVERY AND TARGETING OF THERAPEUTIC AGENTS, (1998) 5/4 (275-280). REFS: 12 ISSN: 1071-7544 CODEN: DDELEB, UNITED STATES, XP002099111 see the whole document --- -/--	1-68



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/26823

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GONZALEZ J.L. ET AL: "Psychological responses to the needle - free injection of insulin with the disposable front-end Medi-Jector.RTM. (MJ-6)." TODAY'S THERAPEUTIC TRENDS, (1998) 16/1 (53-71). REFS: 21 ISSN: 0741-2320 CODEN: TTTRDH, UNITED STATES, XP002099112 see the whole document ---	1-68
Y	WO 96 40958 A (BAYLOR COLLEGE MEDICINE) 19 December 1996 cited in the application see the whole document ---	1-68
Y	FELGNER P L ET AL: "LIPOFECTION: A HIGHLY EFFICIENT, LIPID-MEDIATED DNA-TRANSFECTION PROCEDURE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, November 1987, pages 7413-7417, XP002015347 cited in the application see the whole document ---	1-68
Y	CURIEL D T ET AL: "HIGH-EFFICIENCY GENE TRANSFER MEDIATED BY ADENOVIRUS COUPLED TO DNA-POLYLYSINE COMPLEXES" HUMAN GENE THERAPY, vol. 3, no. 2, 1 April 1992, pages 147-154, XP000579759 cited in the application see the whole document ---	1-68
Y	WAGNER E ET AL: "TRANSFERRIN-POLYCATION CONJUGATED AS CARRIERS FOR DNA UPTAKE INTO CELLS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, 1 May 1990, pages 3410-3414, XP002002759 cited in the application see the whole document ---	1-68
Y	WO 90 11092 A (VICAL INC ;WISCONSIN ALUMNI RES FOUND (US)) 4 October 1990 cited in the application see the whole document ---	1-68
Y	WO 94 24983 A (RIBOZYME PHARM INC) 10 November 1994 cited in the application see the whole document -----	1-68

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-7,21-27,32-38,43-46,51-55,60-64 {partially},  
Claims 8-20,28-31,39-42,47-50,56-59,  
65-68 {completely}

A method for delivering a nucleic acid molecule to a mammal comprising the step of providing said nucleic acid molecule with a transfection facilitating agent through and/or to the skin of said mammal by use of a needle-free device configured and arranged to cause aerosol delivery of said nucleic acid molecule through and/or to the skin of said mammal, kits for carrying out said method, method of making said kit,

2. Claims: 1-7,21-27,32-38,43-46,51-55,60-64 {partially},  
and Claims 69 to 79 {completely}

Method of formulating nucleic acid molecules with modified inert particles comprising the steps of combining said nucleic acid molecules with said modified inert particles wherein said modified inert particles are gold particles and a method for delivering a nucleic acid molecule to a mammal comprising the step of providing said nucleic acid molecule formulated with modified gold particles through and/or to the skin of said mammal by use of a needle-free device configured and arranged to cause aerosol delivery through and/or to the skin of said mammal.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 26823

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-20, 43-68 and 75-79 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see FURTHER INFORMATION, subject 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Appl. Application No

PCT/US 98/26823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9424263 A	27-10-1994	AT 148497 T	15-02-1997
		AU 674742 B	09-01-1997
		AU 6435194 A	08-11-1994
		BG 61993 B	30-12-1998
		BG 100047 A	30-04-1996
		BR 9406455 A	02-01-1996
		CA 2159452 A	27-10-1994
		CN 1120852 A	17-04-1996
		CZ 9502608 A	15-05-1996
		DE 69401651 D	13-03-1997
		DE 69401651 T	15-05-1997
		DK 693119 T	28-07-1997
		EP 0693119 A	24-01-1996
		EP 0734737 A	02-10-1996
		ES 2098131 T	16-04-1997
		FI 954788 A	06-10-1995
		GR 3022939 T	30-06-1997
		HK 1000351 A	06-03-1998
		HU 73516 A,B	28-08-1996
		JP 8509604 T	15-10-1996
		LV 11833 A	20-08-1997
		LV 11833 B	20-12-1997
		NO 953994 A	06-10-1995
		NZ 263606 A	22-08-1997
		PL 311005 A	22-01-1996
		SG 48696 A	18-05-1998
		SI 693119 T	31-10-1997
		SK 124895 A	08-01-1997
		US 5630796 A	20-05-1997
		ZA 9402442 A	10-04-1995
WO 9620732 A	11-07-1996	AU 4743196 A	24-07-1996
		EP 0800403 A	15-10-1997
		JP 10512243 T	24-11-1998
WO 9634967 A	07-11-1996	US 5766901 A	16-06-1998
		CA 2219091 A	07-11-1996
		EP 0826059 A	04-03-1998
WO 9640958 A	19-12-1996	AU 705035 B	13-05-1999
		AU 5714296 A	30-12-1996
		CA 2222550 A	19-12-1996
		EP 0832269 A	01-04-1998
WO 9011092 A	04-10-1990	AT 165516 T	15-05-1998
		AU 5344190 A	22-10-1990
		CA 2049287 A	22-09-1990
		DE 69032284 D	04-06-1998
		DE 69032284 T	08-10-1998
		EP 0465529 A	15-01-1992
		EP 0737750 A	16-10-1996
		ES 2116269 T	16-07-1998
		JP 4504125 T	23-07-1992
		US 5703055 A	30-12-1997
		US 5580859 A	03-12-1996
		US 5589466 A	31-12-1996
		US 5693622 A	02-12-1997

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/26823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9424983 A	10-11-1994	AU 6714094 A EP 0695186 A	21-11-1994 07-02-1996
-----			